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14. ABSTRACT During past year, we have demonstrated that Aurora-A protects ovarian cancer cells from apoptosis induced by chemotherapeutic agent and activates Akt pathway in a p53-dependent manner. Ectopic expression of Aurora-A renders cells resistant to cisplatin (CDDP), etoposide and paclitaxel-induced apoptosis and stimulates Akt1 and Akt2 activity in wild-type p53 but not p53-null ovarian cancer cells. Aurora-A inhibits cytochrome C release and Bax conformational change induced by CDDP. Knockdown of Aurora-A by RNAi sensitizes cells to CDDP-induced apoptosis and decreases phospho-Akt level in wild-type p53 cells. Reintroduction of p53 decreases Akt1 and Akt2 activation and restores CDDP sensitivity in p53-null but not p53-null-Aurora-A cells. Inhibition of Akt by small molecule inhibitor, API-2, overcomes the effects of Aurora-A-on cell survival and Bax mitochondrial translocation. In addition, we have identified several Aurora-A inhibitors by screening ChemDiv library. Moreover, we generated MISIIR-Aurora-A and MMTV-Aurora-A transgenic mice. While MISIIR-Aurora-A mice failed to develop ovarian tumor, MMTV-Aurora-A mice exhibited ductal carcinoma in situ.					
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Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusions.....	7
References.....	7
Appendices.....	8

Introduction

Three tasks have been proposed in this project: 1) examine the clinical/pathological significance and the mechanism of Aurora-A overexpression/activation in ovarian cancer; 2) determine the role of overexpression/activation of Aurora-A in OSE cell transformation *in vitro* and *in vivo* and 3) examine the role of overexpression/activation of Aurora-A in chemoresistance and as a target for ovarian cancer intervention.

Body:

During the last budget year, we have demonstrated that Aurora-A activates Akt pathway leading to ovarian cancer cells resistant to apoptosis induced by cisplatin, taxol and VP16 in a p53 dependent manner. Furthermore, we have screened ChemDiv library using high-throughput assay and identified a number of lead compounds that inhibit Aurora-A kinase. In addition, we have tested Aurora-A oncogenic activity *in vivo* by generating ovarian specific promoter MISIR-Aurora-A and MMTV-Aurora-A transgenic mice. While MISIR-Aurora-A mice failed to develop ovarian tumor, MMTV-Aurora-A mice exhibited ductal carcinoma *in situ*.

1. Aurora-A induces cell survival and chemoresistance by activation of Akt through a p53-dependent manner in ovarian cancer cells.

We have previously shown that Aurora-A is frequently altered in human ovarian cancer (1). Overexpressing Aurora-A induces centrosome amplification and G2/M cell cycle progression. Recently, we reported that Aurora-A protects ovarian cancer cells from apoptosis induced by chemotherapeutic agent and activates Akt pathway in a p53-dependent manner. Ectopic expression of Aurora-A renders cells resistant to cisplatin (CDDP), etoposide and paclitaxel-induced apoptosis and stimulates Akt1 and Akt2 activity in wild-type p53 but not p53-null ovarian cancer cells. Aurora-A inhibits cytochrome C release and Bax conformational change induced by CDDP. Knockdown of Aurora-A by RNAi sensitizes cells to CDDP-induced apoptosis and decreases phospho-Akt level in wild-type p53 cells. Reintroduction of p53 decreases Akt1 and Akt2 activation and restores CDDP sensitivity in p53-null but not p53-null-Aurora-A cells. Inhibition of Akt by small molecule inhibitor, API-2, overcomes the effects of Aurora-A on cell survival and Bax mitochondrial translocation (2, 3). Taken collectively, these data indicate that Aurora-A activates Akt and induces chemoresistance in a p53-dependent manner and that inhibition of Akt may be an effective means of overcoming Aurora-A-associated chemoresistance in ovarian cancer cells expressing wild-type p53.

2. Aurora-A induces mammary tumor but not ovarian carcinoma in transgenic mice model.

Accumulated studies suggest that Aurora-A may play a pivotal role in human oncogenesis (4-6). To examine Aurora-A oncogenic activity *in vivo*, we have generated ovarian specific promoter MISIR-Aurora-A and mammary specific promoter MMTV-Aurora-A transgenic mice. During 18 months' observation, MISIR-Aurora-A mice did not develop ovarian tumor. However, the breast ducts of the MMTV-HA-Aurora-A mice exhibit progressive changes from normal, fibrocystic alteration, simple, complex and atypical hyperplasia to microscopic ductal carcinoma *in situ* (Fig. 1), suggesting the important role of Aurora-A in breast cancer development. The mechanism by which MISIR-Aurora-A mice failed to develop tumor could be due to weak promoter. Further study of the

role of Aurora-A in ovarian oncogenesis is required for crossing MISIIR-Aurora-A mice with other transgenes including p53 and Her2/Neu mice.

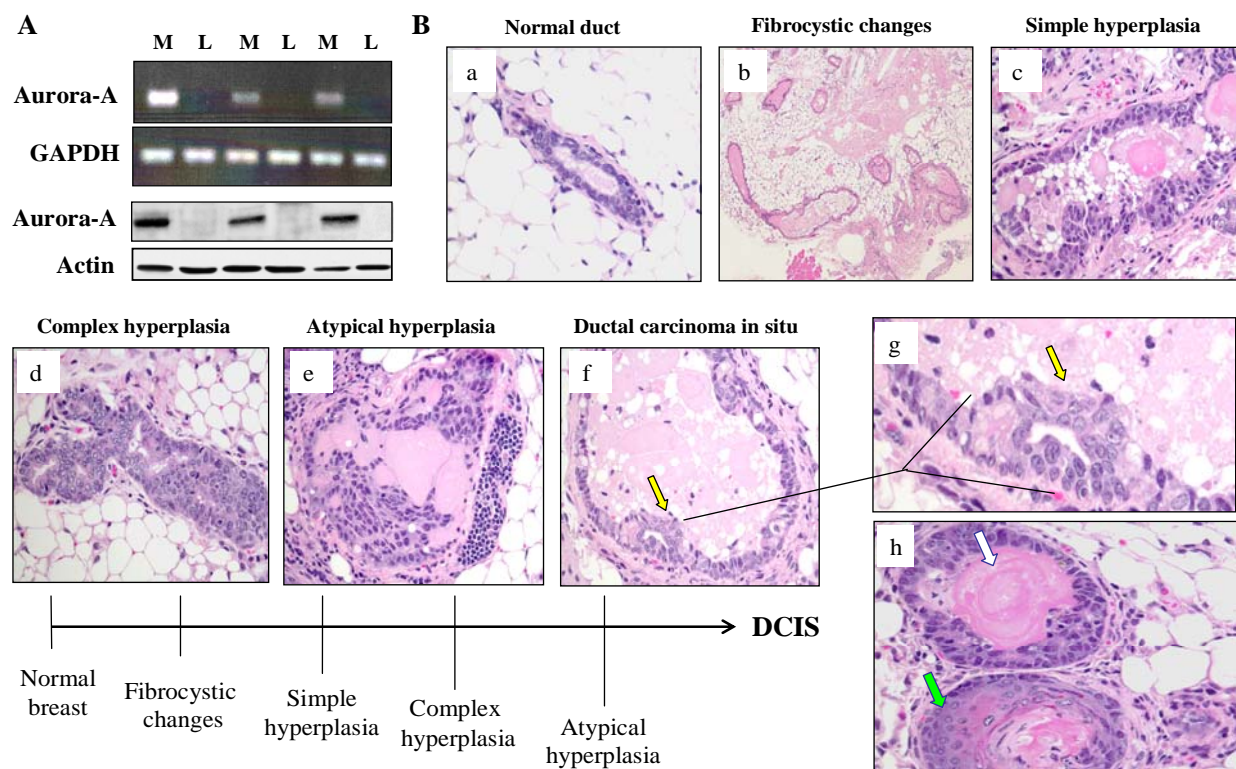


Fig. 1 MMTV-WT-HA-Aurora-A transgenic mice develop DCIS in 18 months. (A) Expression of Aurora-A in mammary gland. Mammary gland (M) and liver (L) were taken from 10-week old transgenic mice and subjected into RT-PCR (panels 1 and 2) and Western blot (panels 3 and 4) analyses. Anti-HA antibody was used for probing Western blot (panel 3). (B) Progressive changes in ductal epithelial cells of the transgenic mice. Histological sections of mammary gland tissue from a 3-month (a), 6-month (b), 9 month (c), 12-month (d), 15-month (e) and 18-month (f and g) old MMTV-WT-HA-Aurora-A mice shows the changes from normal ductal epithelial cells to DCIS. Unlike hyperplasia, DCIS is characterized by the proliferation of highly dysplastic ductal cells, lacking a rim of myoepithelial, with central necrosis and early cribriforming (roman bridges). The tumor cells are uniform and there is no cell-overlapping. (h) Squamous metaplasia (green arrow) and necrosis (white arrow) are observed in the ducts of latent mice.

3 Further Identification of Aurora-A inhibitors.

We have previously identified four potential Aurora kinase inhibitors, RPM215, RPM219, RPM223 and ZK424 through screening of a small focus chemical compound library established at H. Lee Moffitt Cancer Center. In order to obtain Aurora-A specific inhibitor(s), high throughput screening has been performed with ChemDiv library, which is composed of 200,000 compounds. More than 20 lead compounds were identified, one of which is very potent with IC₅₀ 3.8 nM. We are currently testing their specificity.

Key Research Accomplishment

- 1 Aurora-A activates Akt cell survival pathway.

- 2 Expression of Aurora-A induces chemoresistance in human ovarian cancer in a p53-dependent manner.
- 3 Akt inhibitor could partially overcome Aurora-A-induced cisplatin resistance.
- 4 Aurora-A transgenic mice develop breast cancer but not ovarian tumor.

Reportable Outcomes

Publication:

1. Yang H, He L, Kruk P, Nicosia SV, Cheng JQ. Aurora-A Induces Cell Survival and Chemoresistance by Activation of Akt through a p53-dependent Manner in Ovarian Cancer Cells. *Int. J. Cancer*. 119:2304-12, 2006.
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6. Qi Wang, George Z. Cheng, Qiyuan Liu, Santo V. Nicosia, Jin Q. Cheng. Akt activates Twist transactivation towards the epithelial-mesenchymal transition markers through a phosphorylation-dependent mechanism. 97th American Association for Cancer Research Annual Meeting, 2006.
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Conclusion

1. Aurora-A activates Akt pathway.
2. Aurora-A induced chemoresistance depends upon p53 status.
3. Akt inhibitor partially abrogates Aurora-A-induced cisplatin resistance.
4. Aurora-A induces mammary tumor formation in transgenic mice.

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Appendices

Yang H, He L, Kruk P, Nicosia SV, Cheng JQ. Aurora-A Induces Cell Survival and Chemoresistance by Activation of Akt through a p53-dependent Manner in Ovarian Cancer Cells. *Int. J. Cancer*. 119:2304-12, 2006.

Aurora-A induces cell survival and chemoresistance by activation of Akt through a p53-dependent manner in ovarian cancer cells

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Aurora-A is frequently altered in epithelial malignancies. Overexpressing Aurora-A induces centrosome amplification and G2/M cell cycle progression. We have previously shown elevated level of Aurora-A in ovarian cancer and activation of telomerase by Aurora-A in human mammary and ovarian epithelia. Here we report that Aurora-A protects ovarian cancer cells from apoptosis induced by chemotherapeutic agent and activates Akt pathway in a p53-dependent manner. Ectopic expression of Aurora-A renders cells resistant to cisplatin (CDDP), etoposide and paclitaxel-induced apoptosis and stimulates Akt1 and Akt2 activity in wild-type p53 but not p53-null ovarian cancer cells. Aurora-A inhibits cytochrome C release and Bax conformational change induced by CDDP. Knockdown of Aurora-A by RNAi sensitizes cells to CDDP-induced apoptosis and decreases phospho-Akt level in wild-type p53 cells. Reintroduction of p53 decreases Akt1 and Akt2 activation and restores CDDP sensitivity in p53-null but not p53-null-Aurora-A cells. Inhibition of Akt by small molecule inhibitor, API-2, overcomes the effects of Aurora-A on cell survival and Bax mitochondrial translocation. Taken collectively, these data indicate that Aurora-A activates Akt and induces chemoresistance in a p53-dependent manner and that inhibition of Akt may be an effective means of overcoming Aurora-A-associated chemoresistance in ovarian cancer cells expressing wild-type p53.

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Key words: aurora-A; Akt; p53; API-2; chemoresistance; ovarian cancer; kinase; apoptosis; cisplatin

Aurora-A (also named BTAK, STK15, aurora-2, ARKI, AIKI) is a serine/threonine protein kinase, which belongs to the *Drosophila* aurora and *Saccharomyces cerevisiae* Ipl1 (Aurora/Ipl1p) kinase family.¹ Aurora-A is located the chromosomal region (20q13.2) where is frequently amplified in epithelial cancers.² It has been shown that 20q13.2 amplifications involving the Aurora-A gene occur in as many as 12–50% of ovarian, breast, colorectal, pancreatic, bladder, gastric and nonendometrioid cancers.^{3–10} Moreover, up to 50% of ovarian and breast cancers show overexpression and/or activation of Aurora-A, even though the Aurora-A gene amplification is not detected.^{4,11} In addition, ectopic expression of Aurora-A in murine fibroblasts as well as mammary epithelia induces centrosome amplification, aneuploidy and oncogenic phenotype.^{12,13} These studies indicate that Aurora-A plays a critical role in malignant transformation.

Although the mechanism underlying the transforming activity of Aurora-A is not fully understood. Aurora-A may transform cells by regulating the centrosome. Overexpression of Aurora-A has indeed been correlated with centrosome amplification, which can be a driving cause of genomic instability in tumor cells.^{1,2} In addition, recent work has demonstrated that Aurora-A plays an active function in promoting entry into mitosis by regulating local translation of centrosomal stored mRNA, such as cyclin B1.²

We have recently demonstrated that Aurora-A upregulates c-Myc and induces telomerase activity in ovarian and breast epithelial cells.¹⁴ In the current study, we demonstrate that elevated Aurora-A contributes to chemoresistance by activation of Akt pathway in a p53-dependent manner. Aurora-A inhibits cytochrome C release and Bax conformational change. Inhibition of Akt by a small molecule inhibitor API-2 abrogates the effects of Aurora-A. These results

suggest that Aurora-A, in addition to promoting G2-M transition, induces cell survival by cross-talk with Akt pathway.

Material and methods

Reagents and plasmids

Anti-Aurora-A antibody was generated by immunization of rabbit with a previously described GST-Aurora-A/box-2 fusion protein.⁴ Anti-HA and phospho-Akt-S473 and -T308 antibodies were from Roche and Cell Signaling, respectively. Expression plasmids of HA-tagged Aurora-A, Akt1, Akt2 and HA-p53 were described previously.^{14–16} PTEN-Luc construct was kindly provided by Vuk Stambolic (Ontario Cancer Institute, Canada). CDDP, VP16, doxorubicin and paclitaxel were from Sigma.

Cell lines, cell culture, transfection and treatment

The human ovarian epithelial cancer cell lines, A2780S, A2780CP and OV2008, were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum. Transfection was performed using LipofectAmine Plus (Invitrogen). The cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells per dish. After 24 hr, cells were treated with CDDP (10 μ M), paclitaxel (100 nM), VP16 (5 μ M) or doxorubicin (2 μ M) for appropriate time as noted in figure legends.

RNA interference

The 21-mer sense and antisense strands of RNA oligonucleotides were designed and synthesized by Dharmacon Research. The sequence of the Aurora-A RNAi duplexes is ⁹⁷⁹AAATGCCCTGTCTTA-CTGTCA¹⁰⁰⁰. The control (scramble) RNAi (GCGCGCTTTGTAGGATTTCG) was purchased from Dharmacon Research. The RNAi duplexes were reconstituted to 20 μ M in sterile RNase-free water. Transfection of RNAi was performed using Oligofectamine.¹⁴

Immunoprecipitation and immunoblotting and in vitro kinase assay

Immunoprecipitation, immunoblotting and kinase assay were performed as previously described.¹⁷ Briefly, cell lysates were subjected to immunoprecipitation with appropriate antibody. The immunoprecipitates were either immunoblotted with antibody as indicated in the figure legends or used for *in vitro* kinase assay. The kinase reactions were carried out in a kinase buffer.¹⁷ Histone H2B was used as exogenous substrate. The reactions were separated by SDS-PAGE. The dried gel was exposed to a phosphor-screen and the radioactivity of the bands was visualized and quantified by a PhosphorImager using Imagequant software (Molecular Dynamics).

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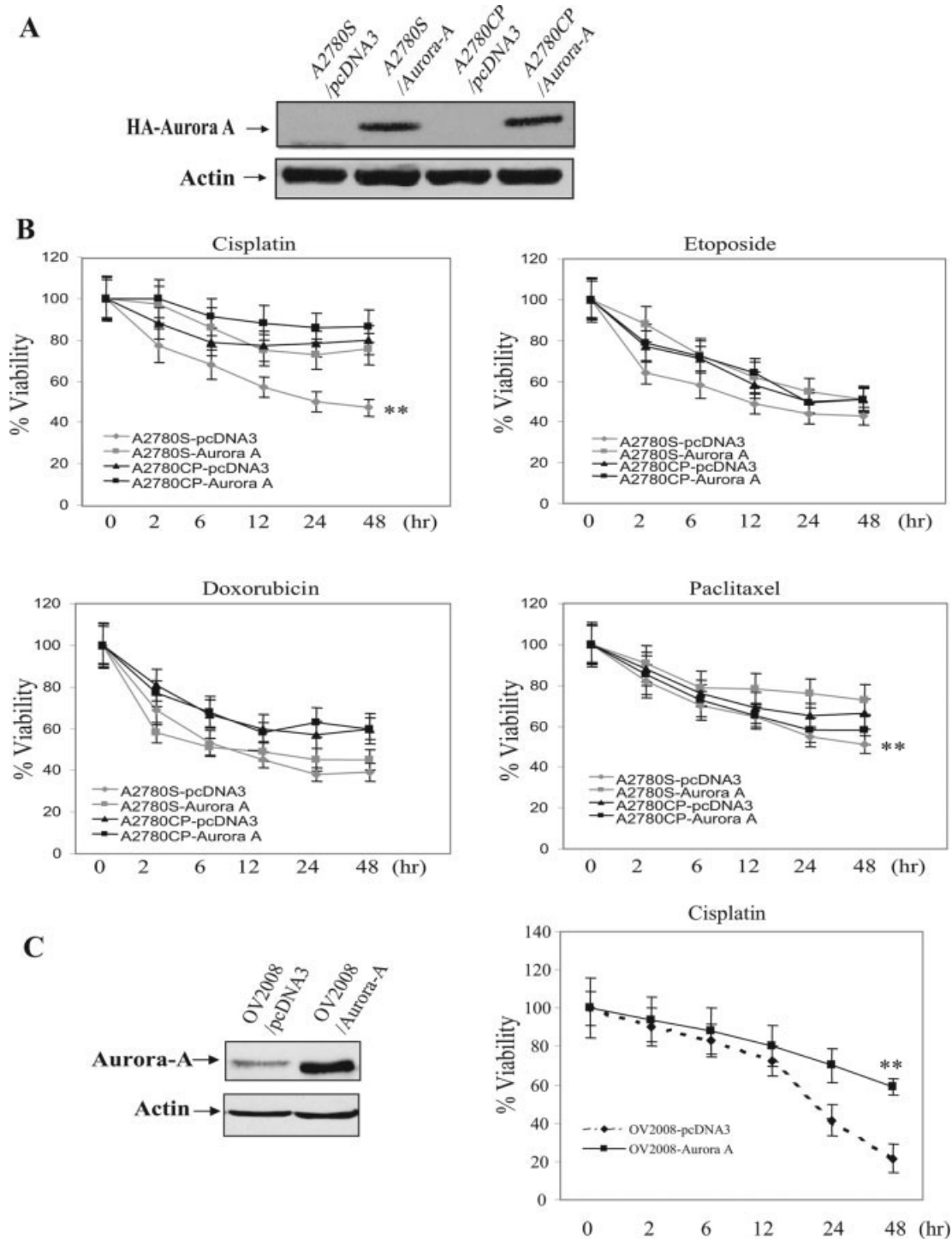


FIGURE 1 – Ectopic expression of Aurora-A induces chemoresistance in wild-type p53 ovarian cancer cells. (a) Stable transfection of Aurora-A. A2780S (wild-type p53) and A2780CP (mutant p53) cells were stably transfected with HA-Aurora-A or pcDNA3. Cell lysates were immunoblotted with anti-HA (upper) and anti-actin (bottom) antibodies. (b) Aurora-A induces chemoresistance in A2780S but not A2780CP cells. Stably transfected cells were treated with CDDP (10 μ M), taxol (100 nM), VP16 (5 μ M) or doxorubicin (2 μ M) for indicated times and then analyzed with MTT assay. Each experiment was repeated 3 times and the results represent the average value. (c) Overexpression of Aurora-A rendered OV2008 cells resistant to CDDP-induced cell death. OV2008 cells were stably transfected with Aurora-A or pcDNA and immunoblotted with anti-Aurora-A antibody (left). The transfected cells were treated with CDDP (15 μ M) for indicated times and the cell death was analyzed as described in panel B (right). (d) Ectopic expression of wild-type p53 restores CDDP sensitivity in A2780CP-pcDNA3 but not A2780CP-Aurora-A cells. The cells were infected with adenovirus WT-p53 or adenovirus alone and then treated with CDDP or vehicle for 24 hr. Cell viability was detected with MTT assay. The results were obtained from 3 repetitions of the experiment. (e) Aurora-A inhibits CDDP-induced caspase-3 activity in A2780S but not A2780CP cells. After treatment with CDDP, cells were lysed and assayed for caspase-3 activity using EnzChek Caspase-3 Assay kit (Molecular Probes). (f) Aurora-A suppresses CDDP-induced apoptosis. Cells were treated with CDDP (10 μ M) for 24 hr and subjected to TUNEL assay. (g) Aurora-A reduces cytochrome C release. Cells were treated with and without CDDP for 6 hr and stained with DAPI and anti-cytochrome C antibody. ** $p < 0.01$.

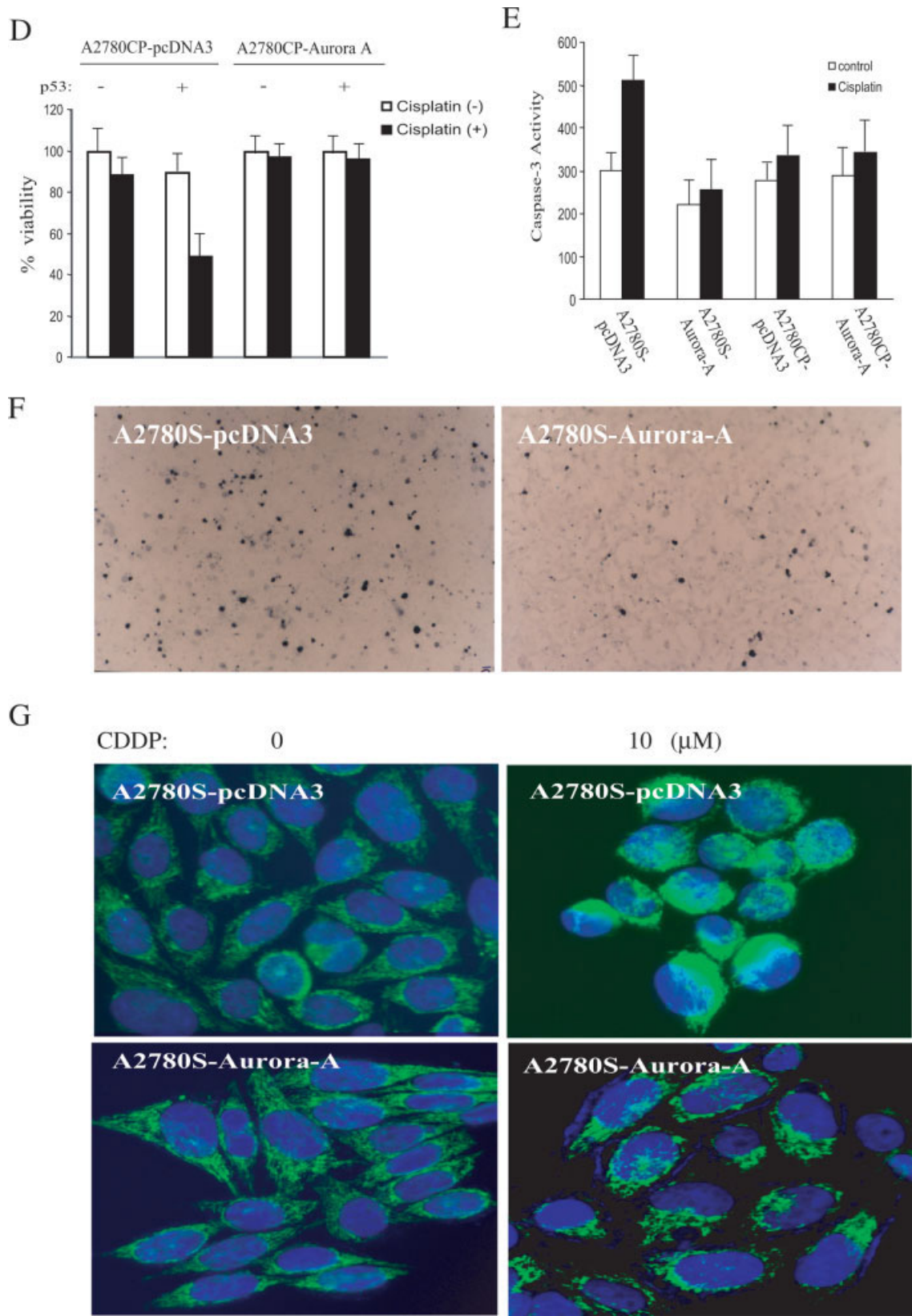


FIGURE 1 – CONTINUED.

Luciferase reporter assay

Cells were seeded in 6-well plate and transfected with PTEN reporter plasmid (pGL3-PTEN-1359/467), pSV2- β -gal, p53 together with or without Aurora-A. After 36 hr of the transfection, luciferase and β -galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix), respectively. Each experiment was repeated 3 times.

Cell survival and caspase-3 activity assays

Cells were treated with 10 μ M CDDP for different times. Apoptosis was determined by fluorescence microscopy analysis (DAPI staining) of nuclear condensation. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as previously described.¹⁸ Caspase-3 activity was measured with the EnzChek Caspase-3 assay kit (Molecular Probes) according to the manufacturer's protocol.

Detection of Bax conformational change

Active Bax was detected by immunoprecipitation with anti-Bax 6A7 antibody, which recognizes only the conformationally changed Bax protein in Chaps lysis buffer. Briefly, cells were lysed in Chaps lysis buffer containing protease inhibitors, and 500 μ g of total protein was incubated with 2 μ g of anti-Bax 6A7 monoclonal antibody in 500 μ l of Chaps lysis buffer at 4°C for 2 hr. Then, 20 μ l of protein G-agarose was added into the reactions and incubated at 4°C for an additional 2 hr, followed by extensive washing in the same lysis buffer and SDS-PAGE/immunoblot analysis with anti-total Bax polyclonal antibody.

Immunofluorescence analysis

The cells were cultured with 25 nM of MitoTracker CMTMRos (Molecular Probes, Inc.) for 30 min prior to fixation in 4% paraformaldehyde and permeabilization in 0.2% Chaps. Immunofluorescent staining was performed with anti-Bax or anti-cytochrome C antibody and FITC-conjugated secondary antibody (1:30, Dako). Nuclei were stained with 0.1 μ g/ml of 4', 6-diamino-2-phenylindole (DAPI) and analyzed by a fluorescence microscopy.

Results

Ectopic expression of aurora-A renders ovarian cancer cells resistant to chemotherapeutic drug-induced apoptosis in a p53-dependent manner

Frequent deregulation of Aurora-A have been observed in human ovarian carcinoma.^{3,4} Ectopic expression of Aurora-A results in malignant transformation.¹² However, the effects of Aurora-A alterations on ovarian tumor cells response to chemotherapeutic drug-induced cell death have not well been documented. To test this, HA-tagged Aurora-A was stably transfected to a pair of ovarian cancer cell lines A2780S and A2780CP, which are derived from A2780 cells and express low levels of Aurora-A.⁴ A2780S contains wild-type p53 and is sensitive to CDDP whereas A2780CP cells carry mutant p53 and resist to CDDP.^{19,20} Expression of transfected HA-Aurora-A was corroborated by immunoblotting analysis (Fig. 1a). The transfected cells were treated in triplicates with CDDP (10 μ M), taxol (100 nM), VP16 (5 μ M) or doxorubicin (2 μ M) for different time points. MTT assay analysis revealed that overexpression of Aurora-A in A2780S induces cells significantly resistant to CDDP-, VP16- and taxol-caused cell death but not to doxorubicin ($p < 0.01$). However, ectopic expression of Aurora-A in A2780CP, as compared to A2780CP-pcDNA3 cells, had no significant effect on cell survival in response to the treatment with 4 different drugs (Fig. 1b) implying that Aurora-A-induced chemoresistance associates with p53 status. To further demonstrate if these findings apply to other cells, OV2008 cells, which carry wild-type p53 and are sensitive to cisplatin, were stably transfected with Aurora-A. Figure 1c shows that overexpression of Aurora-A significantly reduces OV2008 cell death induced by cisplatin ($p < 0.01$).

To examine if p53 is a determinant of Aurora-A-induced chemoresistance, A2780CP and A2780CP-Aurora-A cells were infected with adeno-WT-p53 or control adenovirus. Following 48 hr of incubation, cells were treated with CDDP for different time points and cell survival was assessed by MTT assay. As shown in Figure 1d, reintroduction of p53 restored the CDDP sensitivity in A2780CP but not in A2780CP-Aurora-A cells. On the basis of these findings, we conclude that Aurora-A protects cells from CDDP-induced apoptosis through a p53-dependent manner.

Accumulated studies have established the importance of Aurora-A in G2-M cell cycle progression.¹⁻² As CDDP has been shown to exert its anti-tumor activity largely by activation of intrinsic apoptotic pathway,^{21,22} we next examined whether Aurora-A protects cells from CDDP-induced programmed cell death. The cells were treated with CDDP as described above and analyzed for caspase-3 activity and apoptosis. Consistent with our previous observation,²⁰ approximately 35% of apoptotic cells and elevated caspase-3 activity were detected in pcDNA3-transfected A2780S but not A2780CP cells after 24 hr of treatment with CDDP. However, ectopic expression of Aurora-A in A2780S cells considerably inhibits the caspase-3 activity and apoptosis (Figs. 1e and 1f). Furthermore, cytochrome C release induced by CDDP was also suppressed by Aurora-A (Fig. 1g).

Suppression of Aurora-A expression sensitizes cells to CDDP-induced apoptosis

To further validate Aurora-A involving in CDDP resistance and being a critical target for ovarian cancer intervention, RNAi was employed to knockdown Aurora-A expression in pcDNA3- and Aurora-A-transfected A2780S and A2780CP cells (Fig. 2a). Experiments done in triplicates show that knockdown of Aurora-A enhances CDDP-induced cell death and abrogates Aurora-A-caused CDDP resistance in A2780S cells. In contrast, knockdown of Aurora-A in A2780CP and A2780CP-Aurora-A cells had slightly effects on cell survival (Figs. 2b and 2c). These data suggest that Aurora-A is an important target for anti-tumor drug discovery and further indicate that Aurora-A induces cell survival through p53.

Aurora-A activates Akt

It has been well documented that p53 exerts tumor suppressor function by regulation of cell cycle progression and programmed cell death.^{16,23-25} A major mechanism of p53 proapoptotic function is a transcriptional upregulation of proapoptotic proteins and downregulation of anti-apoptotic molecules. Previous studies have demonstrated that p53 induces PTEN and reduces PIK3CA expression at transcriptional levels leading to inhibition of Akt pathway.^{24,25} We and other have shown that p53 is phosphorylated and inhibited by Aurora-A.^{16,23} Therefore, Akt could be activated by Aurora-A and mediate Aurora-A cell survival signal. To test this hypothesis, phospho-Akt was examined in A2780S and A2780CP transfected with and without Aurora-A. Immunoblotting analysis revealed that the levels of phospho-Akt at residues T308 and S473 were elevated in A2780S-Aurora-A but not A2780S-pcDNA3 cells (Fig. 3a, and data not shown). Notably, Akt was not induced by Aurora-A in A2780CP cells, however, basal level of phospho-S473-Akt is elevated in A2780CP cells due to p53 mutation (Fig. 3a). These data indicate that Aurora-A activation of Akt depends on p53. To further confirm this observation, A2780S, OV2008 and A2780CP cells were transfected with increasing amount of Aurora-A. *In vitro* kinase assay and immunoblotting analysis of Akt1 and Akt2 immunoprecipitates showed that Akt1 and Akt2 kinase activity and phospho-S473-Akt level were induced by Aurora-A in a dose-dependent manner in A2780S and OV2008 but not A2780CP cells. Total Akt1 and Akt2 protein was not affected by Aurora-A (Fig. 3b, and data not shown). Further, suppression of Aurora-A by RNAi decreased phosphorylation levels of Akt in A2780S-Aurora-A but not in A2780CP-pcDNA3 and A2780CP-Aurora-A cells (Fig. 3c). In addition, Akt inhibitor, API-2, abrogated the Akt activation induced by Aurora-A in A2780S

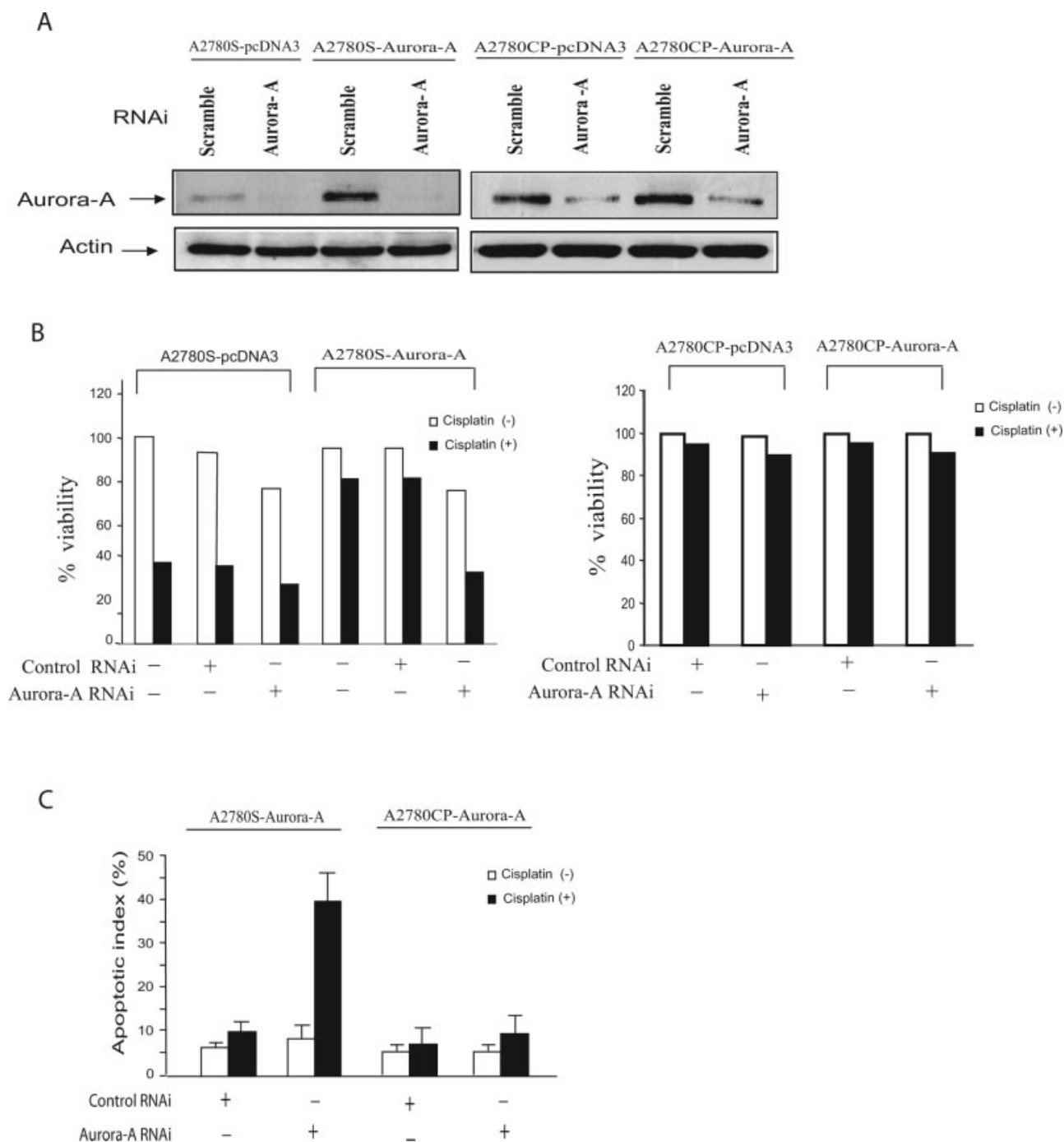


FIGURE 2 – Knockdown of Aurora-A by RNAi sensitizes cells to CDDP-induced apoptosis in a p53-dependent manner. (a) Immunoblotting analysis. Indicated cell lines were transfected with RNAi of Aurora-A or scramble RNAi using oligofectamine. After 48 hr, cells were lysed and immunoblotted with anti-Aurora-A (upper) and anti-actin (bottom) antibodies. (b, c) Cells were treated with RNAi as described in panel A and then exposed to CDDP or vehicle for 24 hr. Cell viability and apoptosis were examined by MTT (b) and TUNEL assays (c), respectively.

as well as in A2780CP cells transfected with or without Aurora-A (Fig. 3d).

Inhibition of Akt abrogates Aurora-A-induced CDDP resistance

As Akt is a major cell survival pathway, we reasoned that Akt mediates Aurora-A-induced CDDP resistance and that Akt inhibitor would override Aurora-A-induced cell survival. We have recently identified a specific Akt inhibitor, API-2/triciribine, by screening

NCI diversity set.²⁶ API-2 exhibits anti-tumor activity in the cancer cells in which Akt is aberrantly expressed/activated and is currently in clinic trial.^{26,27} To examine whether inhibition of Akt by API-2 reverses Aurora-A-induced chemoresistance, A2780S, A2780CP and OV2008 cells that were stably transfected with Aurora-A or pcDNA3 were treated with API-2, CDDP, LY294002/CDDP or API-2/CDDP. After treatment for different time points, cell viability was analyzed. Triple experiments showed that API-2 alone considerably reduces cell survival in A2780S-Aurora-A, OV2008-Aurora-

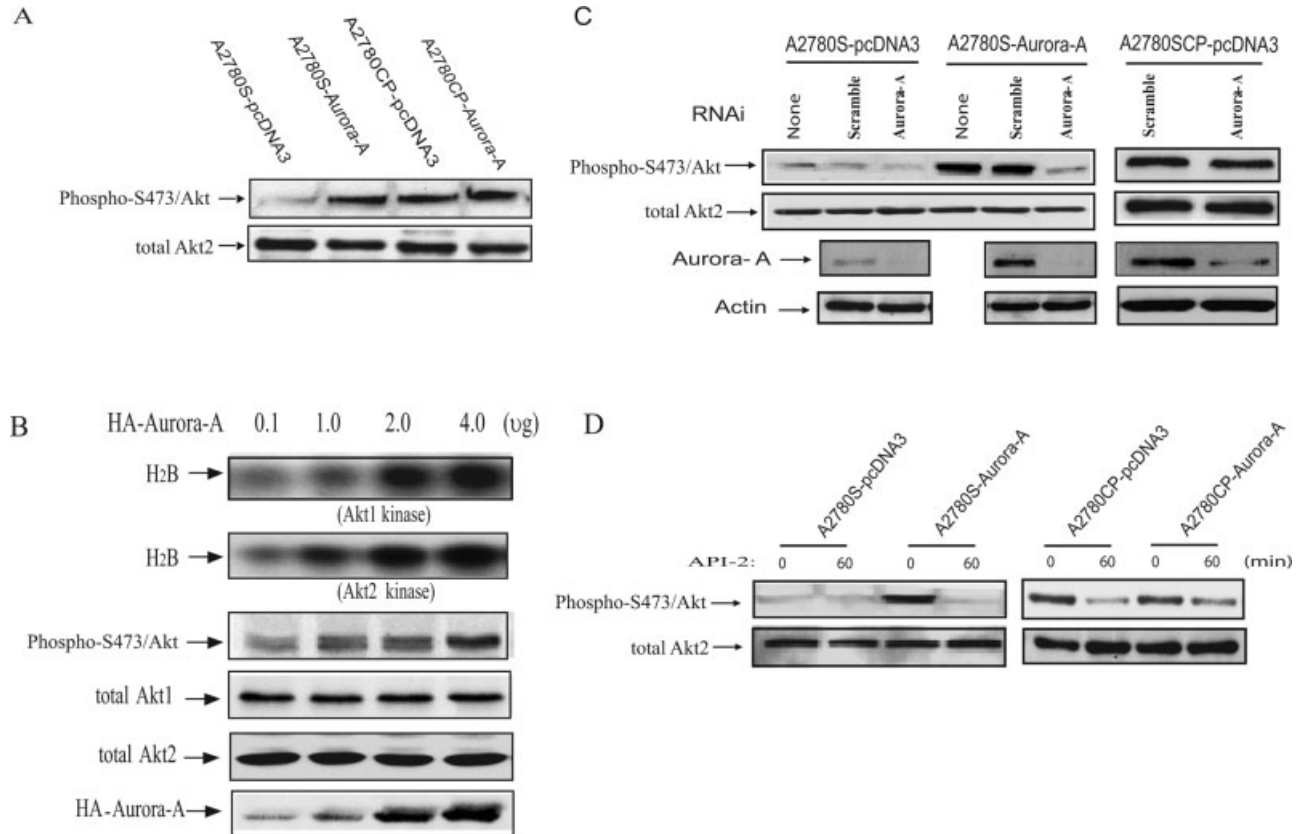


FIGURE 3 – Aurora-A activates Akt. (a) Phospho-Akt is elevated in wild-type p53 A2780S-Aurora-A and mutant p53 A2780CP cells. Indicated cell lines were immunoblotted with anti-phospho-Akt-S473 (upper) and anti-Akt (bottom) antibodies. (b) Aurora-A induces Akt1 and Akt2 kinase activity in A2780S cells. The cells were transfected with indicated amount of HA-Aurora-A and immunoprecipitated with anti-Akt1 and anti-Akt2 antibodies. The immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate (panels 1 and 2). Western blotting analysis was performed with indicated antibodies (panels 3–6). (c) Knockdown of Aurora-A decreases the level of phospho-Akt in wild-type p53 A2780S cells. Following treatment with RNAi of Aurora-A or scramble RNAi for 48 hr, cells were lysed and immunoblotted with indicated antibodies. (d) Akt inhibitor, API-2, reduces phospho-Akt. After treatment with API-2 for 1 hr, cells were lysed and immunoblotted with anti-phospho-Akt-S473 (top) and anti-total Akt (bottom) antibodies.

A and A2780CP cells as compared to A2780S-pcDNA3 and OV2008-pcDNA cells (Fig. 4; $p < 0.05$). Combination of API-2 with CDDP abrogates Aurora-A-induced CDDP resistance. Notably, the combinative treatment reverses the CDDP-resistant phenotype of A2780CP (Fig. 4). Similar result was obtained with PI3K inhibitor LY29004 (data not shown). These data indicate that Aurora-A exerts its cell survival signal largely through activation Akt and inhibition of PI3K/Akt pathway overrides, to a large extent, CDDP resistance induced by Aurora-A.

Aurora-A activation of Akt through inhibition of p53 in A2780 cells

To demonstrate if Aurora-A activation of Akt is indeed resulted from the inhibition of p53 in human ovarian cancer A2780S/A2780CP cells and to further prove that p53 is a determinant of Aurora-A-induced chemoresistance, we examined PTEN protein expression in these cell lines. Wild-type p53 A2780S cells express higher level of PTEN than mutant p53 A2780CP cells do. Aurora-A reduces PTEN expression in A2780S but not A2780CP cells (Fig. 5a). Further, reintroduction of HA-tagged wild-type p53 restores PTEN expression in A2780CP-pcDNA3 but not A2780CP-Aurora-A cells (Fig. 5b), indicating abrogation of p53 function by Aurora-A. We next examined whether Aurora-A inhibits p53 transactivation activity towards PTEN promoter. As shown in Figure 5c, ectopic expression of p53 simulates PTEN promoter activity in A2780CP but fails to transactivate the promoter in A2780CP-Au-

roro-A cells. These data indicate that Aurora-A activates Akt through inhibition of p53-PTEN axis.

As Bax is a major target for p53 (transcriptional level) and Akt (phosphorylation of Bax²⁸) and plays an important role in chemotherapeutic agent-induced apoptosis, we further examined whether Aurora-A reduces Bax conformational change and mitochondrial translocation in response to CDDP. Following treatment of A2780S-pcDNA3 and A2780S-Aurora-A cells with CDDP for 6 and 12 hr, immunoprecipitation and immunostaining were carried out with anti-active Bax antibody (Fig. 5d) and MitoTracker Red fluorescence and anti-Bax antibody (Fig. 5e), respectively. The results show that expression of Aurora-A reduces Bax expression and conformational changes (Fig. 5d) as well as mitochondrial translocation (Fig. 5e). Further, API-2 treatment overrode the Aurora-A-inhibited Bax mitochondrial translocation (Fig. 5e). These data further indicate Aurora-A function beyond its cell cycle control.

Discussion

We have previously demonstrated frequent activation and overexpression of Aurora-A in human ovarian cancer.⁴ In this report, we show that Aurora-A induces chemoresistance and Akt activation in human ovarian cancer cells through inhibition of p53/PTEN cascade. PTEN was downregulated by Aurora-A in a p53-dependent manner. Ectopic expression of Aurora-A abrogates

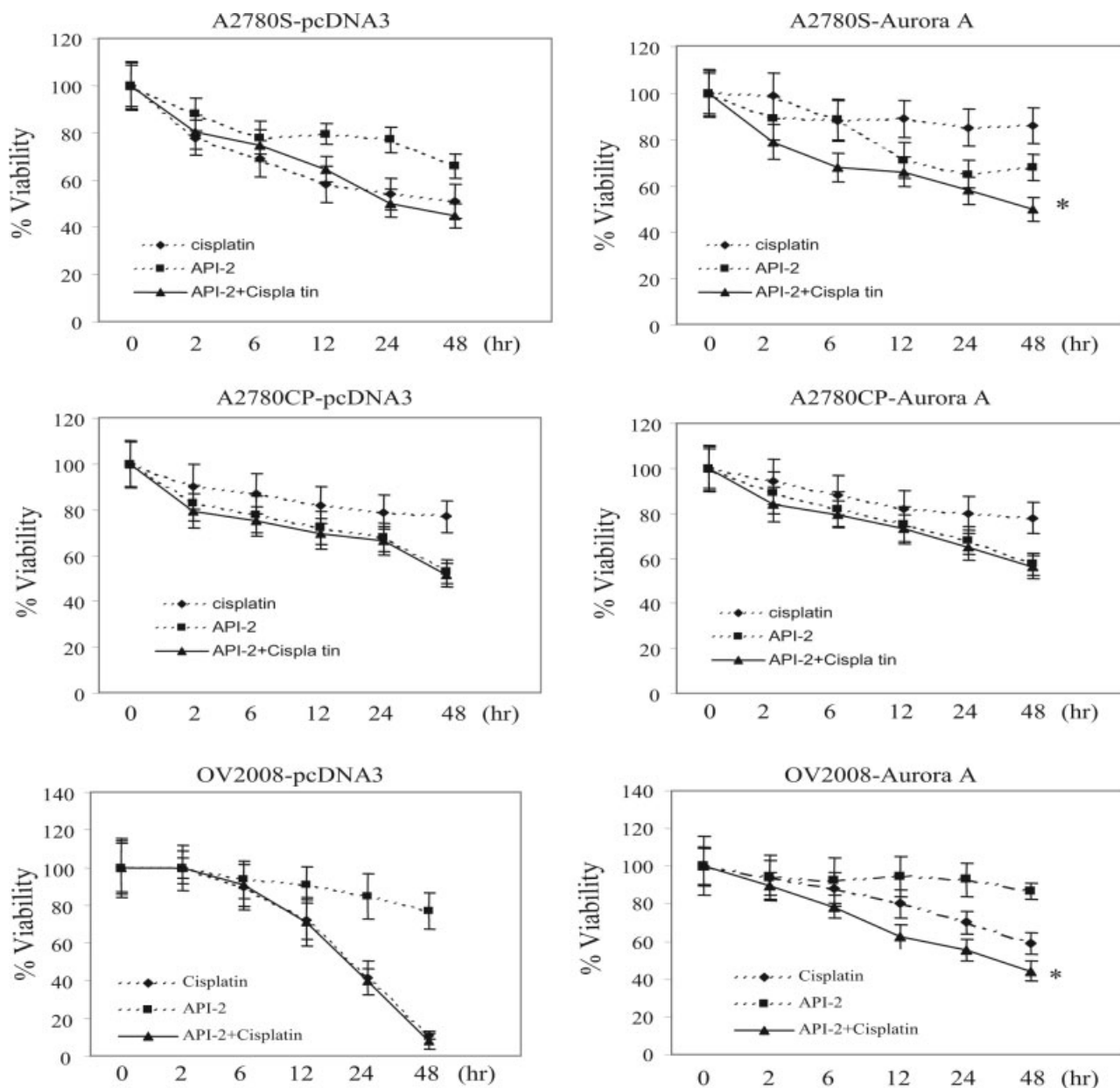


FIGURE 4 – Akt inhibitor, API-2, overrides Aurora-A-induced CDDP resistance. Cells were treated with API-2 (10 μ M), CDDP and API-2/CDDP for indicated times and assayed with MTT. Each experiment was repeated 3 times and the results represent the average value. * $p < 0.05$.

p53-induced PTEN promoter activity, and thus, leads to activation of Akt. Further, Aurora-A-induced chemoresistance was largely overridden by inhibition of Akt pathway. These findings are important for several reasons. First, they provide evidence that Aurora-A not only regulates cell cycle but also cell survival. Second, in addition to paclitaxel, overexpression of Aurora-A also induces cancer cells resistant to other chemotherapeutic drugs (Fig. 1). Third, p53 is a determinant of Aurora-A-mediated chemoresistance. Finally, this is the first identification of the cross-talk between Aurora-A and Akt pathways, and Akt inhibitor largely abrogates Aurora-A-induced cell survival.

Aurora-A locates to the spindle pole during mitosis and is regulated in a cell cycle-dependent manner; its protein is low in G1/S, upregulated during G2/M, and reduced rapidly after mitosis.^{1,2} It has been shown that Aurora-A phosphorylates several proteins that are important for mitosis, including histone H3,^{29,30} a key

molecule in conversion of the relaxed interphase chromatin to mitotic condensed chromosomes; CPEB (cytoplasmic polyadenylation element-binding protein), best known for its role in promoting polyadenylation of cyclin B mRNA³¹; TACC3, a protein required for stabilization and organization of microtubules³²; Eg5, a kinesin-like protein involved in both centrosome separation and spindle assembly and stability³³ and TPX2, which is required to generate stable bipolar spindle.³⁴ In this study, we demonstrate that Aurora-A possesses antiapoptotic function by inhibition of p53 leading to downregulation of PTEN and activation of Akt. While p53 reduces cell survival through regulation of a number of downstream targets and mitochondrial translocation,³⁵ activation of Akt by Aurora-A seems to play a considerable role in Aurora-A-induced CDDP resistance as Akt inhibitor overrides Aurora-A-induced CDDP resistance and some of p53 targets are also regulated by Akt, such as Bax (Fig. 5f).²⁸

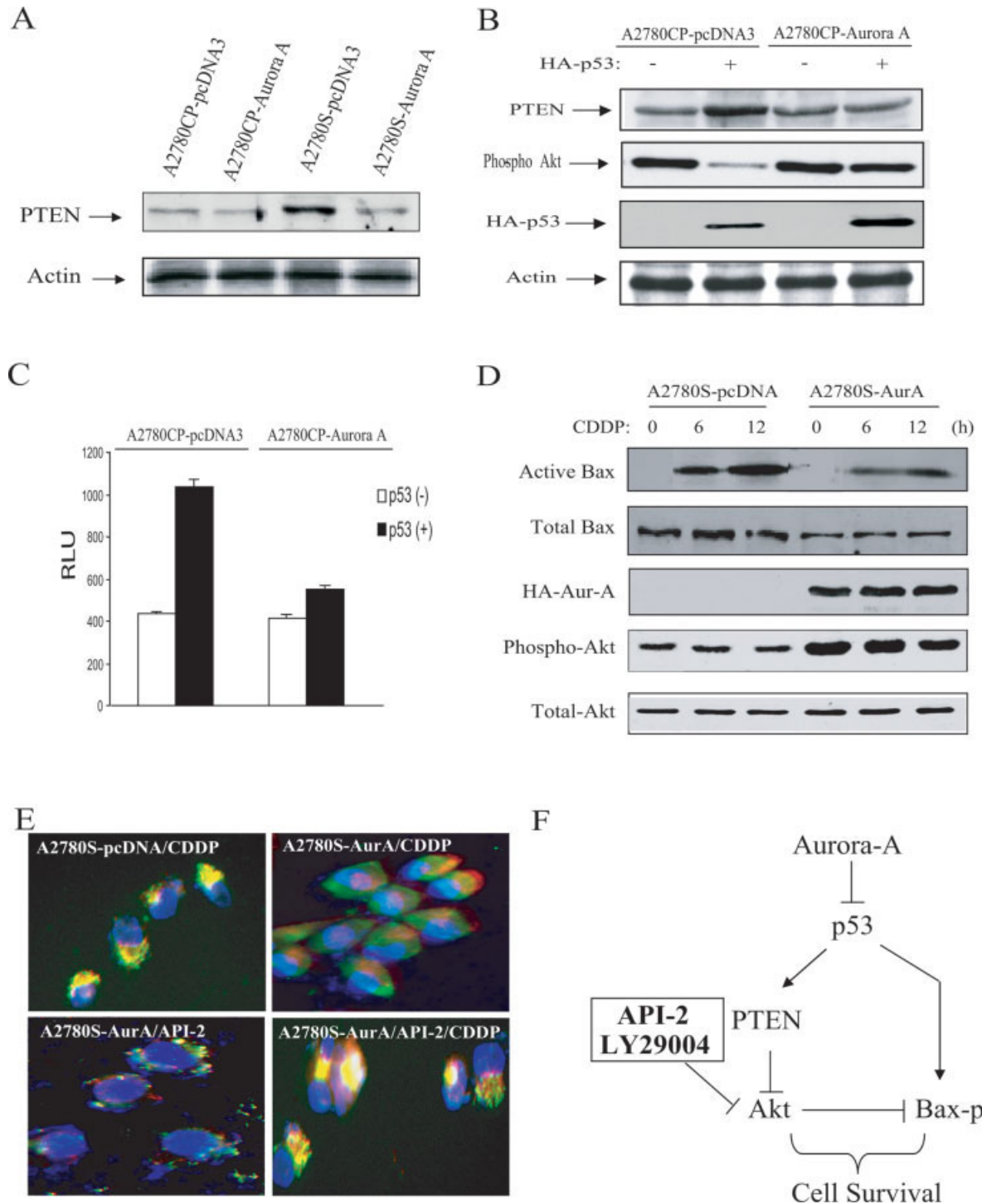


FIGURE 5 – Aurora-A activation of Akt through inhibition of p53-PTEN axis. (a) Expression of PTEN is inhibited by Aurora-A in A2780S but not A2780CP cells. The immunoblot was probed with indicated antibodies. (b) Reintroduction of wild-type p53 induces PTEN expression in A2780CP but not A2780CP-Aurora-A cells. Cells were transfected with HA-p53 (+) or vector alone (–). After 48 hr of incubation, cells were lysed and immunoblotted with indicated antibodies. (c) Activation of PTEN promoter by p53 in A2780CP-pcDNA3 but not A2780CP-Aurora-A cells. Cells were transfected with PTEN-Luc together with or without p53. After 48 hr of transfection, cells were subjected to luciferase reporter assay. (d) Aurora-A reduces Bax conformational change. A2780S-pcDNA3 and A2780S-Aurora-A cells were treated with CDDP for indicated time points. Active Bax was immunoprecipitated with anti-Bax 6A7 antibody and the immunoprecipitates were blotted with anti-total Bax antibody (top). Total cell lysates were immunoblotted with indicated antibodies (panels 2–5). Due to Aurora-A inhibition of p53, total Bax protein was decreased in A2780S-Aurora-A cells, implying that the reduced Bax conformational change was resulted from both activation of Akt²⁸ and decreased Bax expression. (e) Aurora-A inhibits CDDP-induced mitochondrial translocation of Bax, which is overridden by Akt inhibitor, API-2. Cells were treated with CDDP and/or API-2 for 3 hr and stained with DAPI, MitoTracker (red) and anti-Bax antibody (green). Bax mitochondrial translocation was shown in yellow. (f) Schematic illustration of Aurora-A contribution to cell survival by activation Akt and inhibition of Bax through p53.

We have previously shown frequent activation of Akt and elevated Aurora-A in human ovarian cancer.^{4,36} A subset of tumors with activation of Akt could be resulted from overexpression of Aurora-A. Both Akt and Aurora-A are major pathways involved in cell transformation and chemoresistance; therefore, they have been drawn a significant attention for anti-cancer drug discovery. Because Aurora-A activates Akt and because Aurora-A-induced chemoresistance is p53 dependent, inhibition of Akt, besides Aurora-A inhibitors, could be an effective means for overcoming Au-

ror-A-induced chemoresistance in ovarian cancer cells expressing wild-type p53.

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